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Request for grant of a patent

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1	Your reference	MRH/P15464
2	Patent application number	**9900009.3**
3	Full name, address and postcode of the applicant Patents ADP number State of incorporation	ML Laboratories Plc Blaby Hall Church Street Blaby LE8 4FA United Kingdom 7436405001
4	Title of the invention	Gene Therapy
5	Name of agent Address for service Patents ADP number	Harrison Goddard Foote Belmont House 20 Wood Lane Headingley Leeds LS6 2AE 14571001 ✓

6 Priority applications	Country	Priority App No	Date of Filing

7	Parent application (eg Divisional)	Earlier Application No	Date of Filing
8	Statement of Inventorship Needed?	Yes	
9	Number of sheets for any of the following (not counting copies of same document)		
	Continuation sheets of this form		
	Description	7	
	Claims		
	Abstract		
	Drawings		
10	Number of other documents attached		
	Priority documents		
	Translations of priority documents		
	P7/77		
	P9/77		
	P10/77		
	Other documents		
11	I/We request the grant of a patent on the basis of this application.		
	Signature Harrison Goddard Foote	Date 30 Dec 1998	
12	Name and daytime telephone number of person to contact in the United Kingdom	Michael R Harrison	
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GENE THERAPY

This invention relates to a form of gene therapy known as genetically directed enzyme prodrug therapy (GDEPT).

GDEPT is of particular interest with respect to the treatment of cancer in that it offers advantages over conventional chemotherapeutic methods of cancer treatment. In such conventional methods the drugs administered to the patient attack not only the targeted cancer cells but also normal cells. Destruction of cancer cells is achieved at the expense of inflicting damage on normal cells, creating serious side-effects. In treatment of cancer by GDEPT the objective is to create an anti-cancer drug in situ within the cancer cell while creating little or none in normal cells, thereby attacking the cancer cells while leaving the normal cells substantially unaffected. This is achieved by administration to the patient of a vector containing a gene for an enzyme which can convert a relatively non-toxic substance (commonly referred to as a prodrug) into a cytotoxic agent. The vector also contains a promoter, ie a DNA sequence constituting a switch for the gene, this promoter being responsive to a regulatory protein found solely in the cancer cells or to a greater extent in the cancer cells than in normal cells. The gene is thus expressed only (or mainly) in the cancer cells so it is only (or mainly) in the cancer cells that the enzyme is produced and that conversion of the prodrug to the cytotoxic agent takes place. Formation of the cytotoxic agent therefore takes place primarily in the cancer cells. In this way the cancer cells are selectively attacked, with relatively little damage to normal cells.

In one example of the use of GDEPT in cancer treatment, the prodrug is 5-fluoro-cytosine (5-FC). 5-FC is itself relatively non-toxic to human cells but can be converted into a potent anti-cancer drug, 5-fluorouracil (5-FU), by the enzyme cytosine deaminase. A bacterial gene which expresses cytosine deaminase is incorporated in a viral vector in association with a promoter which is responsive to a regulatory protein that is characteristic of the particular type of cancer cell under attack. For instance, in treating breast cancer the promoter could be one which is

responsive to the regulatory protein ERBB2 or in treating liver cancer one which is responsive to alpha-fetoprotein.

In known GDEPT techniques, difficulty has been encountered in achieving as high a degree of selectivity as is desirable, ie in destroying cancer cells while limiting the damage to normal cells. This is at least partly due to the fact that normal cells may come under attack from cytotoxic agents which have been formed in the cancer cells but have found their way out of those cells, for example when the cells break down under the cytotoxic action of the drug.

An object of the present invention is to provide a form of GDEPT in which the prodrug is one which is enzymatically converted to an anti-cancer drug which, though toxic within cancer cells, is readily detoxified upon emergence from such cells.

This and other objects are achieved by use of paracetamol, or a clinically approved analogue thereof, as a prodrug in GDEPT.

Paracetamol is a widely used mild pain reliever and antipyretic. However, it is a potentially dangerous drug in that an overdose of it can cause serious, even fatal, damage to the liver. This is due to the fact that liver cells express a gene for a p450 enzyme, specifically P450 1A2, also to a much lesser extent CYP 2E1 and CYP 3A4. This enzyme can convert paracetamol into a metabolite, N-acetylbenzoquinoneimine (NABQI), which is highly cytotoxic. For standard dosages of paracetamol, the toxicity of NABQI is countered in the liver by conversion of NABQI into a non-toxic substance by reaction with glutathione, a normal component of human cells. The supply of glutathione is however insufficient to deal with the large amounts of NABQI formed in liver cells after an overdose of paracetamol and the cells are therefore then damaged or destroyed.

When paracetamol constitutes the prodrug in GDEPT, the vector administered contains a gene for a p450 enzyme, preferably p450 1A2, and the cytotoxic agent formed in the cancer cells is NABQI. In contrast to other cytotoxic agents, NABQI inflicts little or no damage on normal cells when it finds its way out of the cancer cells because it is rapidly detoxified by reaction with serum albumin and is then readily excreted in the urine.

It might have been expected that the use of paracetamol as a prodrug in GDEPT would be impractical. Selective expression of the gene for the enzyme P450 1A2 in cancer cells could be effected by administration of a vector containing that gene in association with a promoter which is responsive to a regulatory protein found only in the cancer cells. The enzyme P450 1A2, created as a result of the entry into cells of that vector, would then convert paracetamol into NABQI in the cancer cells and damage or destroy them. As in conventional GDEPT using prodrugs other than paracetamol, selectivity between cancer cells and normal cells would be achieved because entry of the vector into normal cells would not cause expression of the P450 1A2 gene contained in the vector since the normal cells do not contain the regulatory protein which activates the chosen promoter for the gene. In general, the normal cells would therefore not contain the enzyme P450 1A2 and would be unaffected by the presence of paracetamol because in such cells it would not undergo intracellular conversion to NABQI. However, as mentioned above, normal liver cells naturally express a gene for P450 1A2. It would therefore be expected that administering a dose of paracetamol high enough to create a level of NABQI in cancer cells capable of killing such cells could also result in the creation of sufficient NABQI in the normal liver cells to kill them too. Surprisingly, this is not so, probably due to a difference in the glutathione content of normal liver cells and that of cancer cells. It appears that most cancer cells may contain only about one-fifth of the glutathione present in normal liver cells. The concentration of the cytotoxic NABQI is therefore kept much lower in the normal liver cells than in the cancer cells because more NABQI can be detoxified, by combination of NABQI with glutathione, in the normal liver cells than in the cancer cells.

The vector used in the present invention is one containing a gene for a p450 enzyme, preferably for P450 1A2, and a promoter which acts as a switch for that gene and which is responsive to a regulator protein characteristic of the type of cancer being addressed. The gene can be derived from human DNA. However, it may be advantageous to use a P450 gene derived from non-human DNA, for example mouse DNA or hamster DNA. The P450 enzyme generated by the mouse gene is relatively unaffected by certain compounds, for example furaphylline, which act as inhibitors of the form of the enzyme P450 1A2 generated by the human gene. Administration of such inhibitors makes it possible to raise the dosage of paracetamol above the normally safe dosage; an inhibitor such as furaphylline can protect the normal liver cells by inhibiting the form of P450 generated by expression in those cells of the human gene, while having little or no effect on the form of P450 generated by expression of the mouse gene in the cancer cells. The level of NABQI in the normal liver cells is therefore diminished by such inhibitors while the level of NABQI in the cancer cells is relatively unaffected by the inhibitors.

In vivo delivery of DNA containing the gene for the p450 enzyme can be effected by both direct injection (in situ delivery) and also systemic delivery, to include intravenous and intraperitoneal delivery. Both viral and non-viral delivery systems may be used.

Viral vectors include the retroviruses, adenovirus, herpes simplex virus, vaccinia virus, adeno-associated virus, epivirus, and lentivirus (to include HIV-1 and equine infectious anaemia virus). Also, the delivery vector may consist of a hybrid of two or more types of vector.

With regard to non-viral vectors, synthetic uptake of DNA into mammalian cells can be facilitated by condensing it with lipids, proteins or peptides. Examples include deridrimers and lipid vectors. Bacteria such as salmonella could be a more novel delivery vehicle. The DNA can also be coated on to microprojectiles and fired into the nuclei or target cells by a gene gun.

To target the tumour cell transcriptional activation can be used. There are many documented in the literature. Typical areas of interest include: tyrosine kinase promoter (TRP-1) for melanoma cells, HER2, ERBB2, ERBB3, HER3, for pancreatic, ovarian, breast and gastro-intestinal tumours. Other examples include CEA for colon tumour cells.

Chimeric transcription elements could also be adapted for delivery, for example MUCI promoter combined with ERBB2.

In a specific example, human cytochrome P4501A2 (CYP1A2) is chosen to convert paracetamol to NABQI. (Other p450 genes that may be usable are CYP2E1 and CYP3A4). The CYP1A2 cDNA is cloned downstream of a 544 bp fragment of the proximal 5' flanking region of the human ERBB2 gene in pBluescript II SK+ and then the chimeric minigene (ERBB2 promoter-CYP1A2 cDNA) is subcloned into a variety of eukaryotic expression vectors including:-

(a) the plasmid pPolyA (based on the commercial vector pcDNA [InVitrogen] from which the CMV promoter has been excised). Transfection is performed by application of plasmid DNA in the presence of cationic liposome complexes, either commercially obtained reagents such as Lipofectin (Life Technologies) or novel experimental agents (Genzyme). In order to allow for selection of genetically transformed clones the ERBB2-CYP1A2 plasmid is cotransfected at a 9:1 molar ratio with pSV2neo which encodes resistance to geneticin.

(b) the double copy retrovirus N2A, which allows conditional expression of the insert outside the transcription unit driven by the retroviral long terminal repeat promoter. Amphotropic retroviral stock is produced by packaging of the virus in GP + env AM12 cells.

(c) the adeno-associated virus vector psub 201, which when cotransfected into adenovirus-expressing cells together with pAAV/Ad leads to production of recombinant AAV that allows conditional expression of the insert in target cells.

The target cells in this example are human breast and pancreatic cancer cell lines which either over-express ERBB2 due to transcriptional upregulation or express normal (undetectable) levels.

The level of expression of CYP1A2 is measured by Western blot analysis utilizing anti-peptide antibodies described in the literature (Edwards RJ et al, Biochem Pharmacol 1993; 46: 213-220 and Murray BP et al, Carcinogenesis 1993; 14: 585-592). These antibodies bind specifically to CYP1A2 in human liver microsomal fraction. In addition, one of the antibodies has been shown to bind readily to CYP1A2 expressed in a human B lymphoblastoid cell line transfected with a plasmid vector expressing human CYP1A2. In these cells the level of expression of human CYP1A2 was similar to that found in human liver, ie 8 pmols per mg protein (Edwards RJ et al, Carcinogenesis 1994; 15: 829-836). Microsomal fractions are prepared from tumour cells transfected with human CYP1A2, or cells transfected with an unrelated gene, eg cytosine deaminase, in the same vectors. Washed cells are disrupted using a Dounce homogeniser and the microsomal fraction prepared by ultracentrifugation and stored frozen at -80°C as described previously. Western blotting of microsomal fractions is performed as described previously (Boobis AR et al, Br J Clin Pharmacol 1980; 9: 11-19) employing enhanced chemiluminescence to maximise sensitivity.

In addition, the functional activity of expressed human CYP1A2 is determined in the tumour cells by measuring the rate of *O*-deethylation of phenacetin. At an appropriate substrate concentration (4 μM) this reaction is specifically catalysed by CYP1A2 in human liver microsomal fraction. Tumour cell microsomal fractions prepared as described above are incubated at 37°C in the presence of NADPH and the production of paracetamol is determined by gas chromotography/negative ion

chemical ionisation mass spectrometry using deuterated paracetamol as internal standard. This highly sensitive assay easily measures CYP1A2 activity in small quantities (<10 μg) of human liver microsomal fraction which typically has an activity of 70 pmols/min/mg protein.

Tumour cells successfully expressing CYP1A2 and control cells (transfected with another gene, eg cytosine deaminase) are exposed to a range of concentrations (0.1 to 10mM) of paracetamol and the following parameters measured:-

(i) Cell viability, by measurement of the release of lactate dehydrogenase and effects on cell proliferation by measurement of mitochondrial enzyme activity (MTS assay, Promega).

(ii) Glutathione depletion. The intracellular glutathione content, comprising reduced and oxidised forms of glutathione is measured using a kinetic assay in which glutathione in the presence of glutathione reductase catalyses the continuous reduction of 5,5'-dithio-bis(2-nitrobenzoic acid) by NADPH. The rate of the reaction is proportional to the concentration of glutathione. The reaction is monitored at 412 nm and quantified by comparison with standards.

(iii) Covalent binding of radioactivity to cell protein following exposure to radioactive paracetamol. Cells are incubated for up to 90 min with [^{14}C]paracetamol. After washing, cellular protein is precipitated with trichloroacetic acid and the precipitate washed extensively with 80% methanol to remove unbound radioactivity. The protein pellet is digested in sodium hydroxide, neutralised, and the bound radioactivity measured by scintillation spectroscopy.

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